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In studies to define the mechanisms involved in the progression of immortal, non-tumorigenic prostate cells to a tumorigenic state, we have found that molecular chaperones are elevated, which causes increased telomerase activity. In order to determine the importance of the chaperone increase during prostate cancer progression, we have taken a 2-pronged approach, using both genetic and pharmacologic approaches: 1-define whether ectopic chaperone expression results in transformation, and 2- determine whether chaperones are targets for prostate cancer therapy. The hsf-1 transcription factor has been over-expressed in non-tumorigenic prostate cells, resulting in increased hsp90 and hsp70 expression, an upregulation of telomerase, and no effect on tumorigenicity. However, preliminary data suggests that hsf-1 may directly effect telomerase expression, which would further define the regulation of telomerase during cancer progression. Using both a pharmacologic (radicicol, a specific hsp90 inhibitor) and genetic (siRNA to hsp90) approaches, prostate cancer cell lines exhibit only a transient decline in telomerase activity but a significant decrease in telomeres, which we have shown to be directly damaged by free radicals produced as a result of deregulation of the nitric oxide synthase pathway.

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Introduction

Telomerase is a cellular reverse transcriptase that is associated with over 90% of human prostate cancers and is composed of 2 integral components, an RNA template (hTR - human Telomerase RNA) and a catalytic polymerase (hTERT - human TElomerase Reverse Transcriptase) (Weinrich et al 1997). Telomerase is an obvious chemotherapeutic target (Shay and Bacchetti, 1997). Telomerase activity requires two core components, hTERT and hTR to be assembled into a functionally active enzyme by the Hsp90 chaperone complex (Holt et. al., 1999). We have previously demonstrated that chaperones are essential for optimal telomerase assembly *in vitro* (Holt et. al., 1999) and that Hsp90 itself remains associated with the functional telomerase complex (Forsythe et. al., 2001) (see Figure 1).

In a human prostate cancer model, increased assembly of telomerase by chaperones, including Hsp90, has been shown to correlate with prostate cancer progression, which is defined as increased aggressiveness *in vivo* (Akalin et. al., 2001). These findings indicate that increased expression of the Hsp90 chaperone complex with the associated activation of telomerase activity may be important steps in prostate cancer formation (Holt et. al., 1999; Akalin et. al., 2001). While telomerase in cancer progression has been widely studied (reviewed by Shay and Bacchetti, 1997), the role of chaperones in carcinogenesis and their interplay between telomerase and its substrate, the telomere, are less well defined.

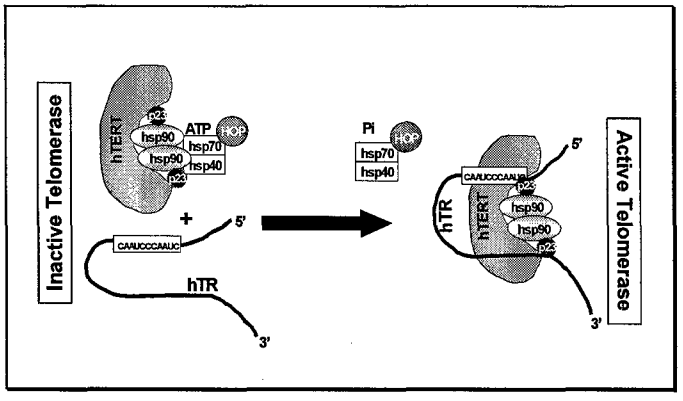

Figure 1. The hsp90 complex is required for assembly of active telomerase. Our working model for the chaperone-mediated ordered assembly of active human telomerase. [hTR - human telomerase RNA; hTERT - human telomerase reverse transcriptase]

Body

Normal human prostate epithelial cells were immortalized by expression with the SV40 large T antigen oncogene, and an immortal, non-tumorigenic, telomerase-positive cell line was selected, P69. When injected into nude mice, no tumors formed within the standard 8-12 weeks, but if left in the animal for 6 months, 2 palpable sporadic tumors from a total of 19 mice formed after in vivo selection (Bae et al., 1994) (Figure 2). Both lines, M2205 and M2182, were propagated in culture and reinjected into mice and were found to be tumorigenic. After undergoing another round of selection, the metastatic subline, M12, was generated. As our model for prostate cancer, the P69-M12 progression scheme provides an excellent system from a defined genetic background to study the molecular and cellular changes that occur during prostate cancer progression. Having found elevated telomerase levels in the more advanced prostate cancer lines and tumor samples (Akalin et al., 2001), our data indicates that this change in activity is due to an increase chaperone-mediated telomerase assembly rather than expression of the hTERT and hTR core components of the telomerase holoenzyme. As such, our goals are to determine if

chaperones are the cause of the transformation event during prostate cancer progression and to show that chaperones are likely targets for anti-telomerase therapy in advanced prostate cancer.

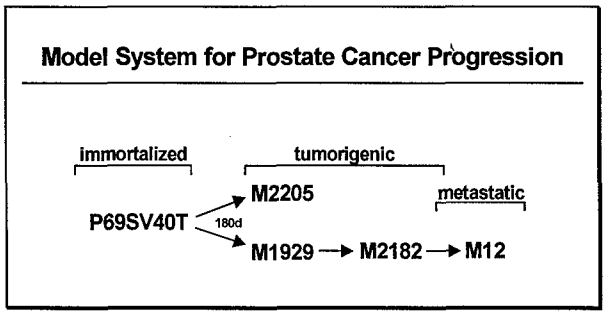

Figure 2. Prostate cancer progression model system. In our *in vitro* model of prostate cancer progression, as cells convert from immortal to tumorigenic and eventually metastatic capabilities, both telomerase activity and global chaperone protein levels increase.

***Objective #1*: Define the role of chaperones and telomerase activation during prostate cancer progression.**

This first aim is designed to determine if the elevated chaperone expression levels can cause transformation in the non-tumorigenic P69 cells. We have designed and made retroviral constructs for chaperone-related genes, hTERT, and oncogenic ras. Each construct can be utilized and stably selected into cells either alone or in combination with any of the other constructs, which has now been done for each as reported below.

We reported last year that infection and stable selection of hsp90 showed a modest, but reproducible increase in telomerase activity. We have also ectopically expressed the p23 co-chaperone in the P69 nontumorigenic cells and show an increase in p23 without a concomitant increase in telomerase activity, suggesting that p23 is not limiting in these cells (Figure 3). We also find that there is no effect on hsp90 when p23 is over-expressed, although last year we reported that ectopic expression of hsp90 showed a modest increase in p23 levels.

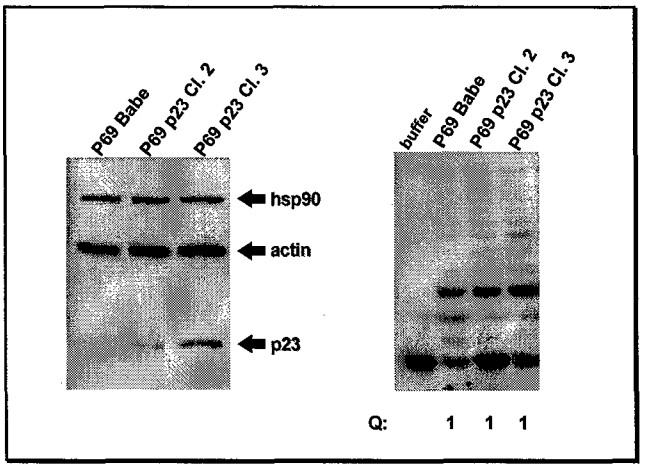

Figure 3.Effects of over-expression of the p23 co-chaperone. Retroviral expression of p23 in P69 cells shows an increase in protein levels without changes in other chaperone-related proteins and no significant differences in telomerase activity.

Over-expression of hsf-1 in P69 cells results in an increase in hsp90, hsp70, hsp27, and hsf-1 (Figure 4 and data not shown), and importantly for our studies, telomerase activity is also reproducibly increased in these cells after hsf-1 expression (Figure 4). Certainly, there is variability in telomerase activity levels in individual clones for hsf-1, but this variability and the range of activity levels are significantly upregulated compared P69 clones (data not shown).

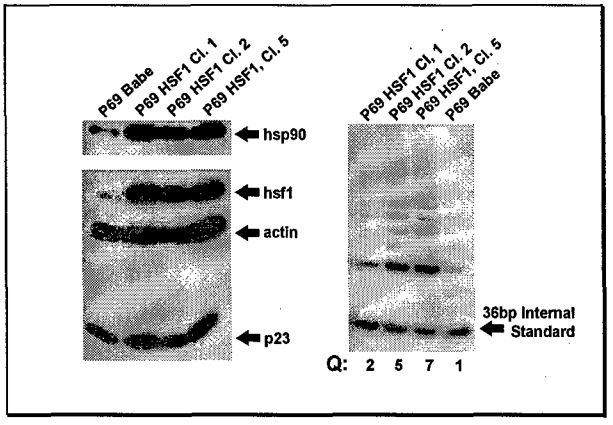

Figure 4. Effects of hsf-1 overexpression in P69 cells. Over-expression of the heat shock transcription factor, hsf-1, induces expression of hsp90 with a modest increase in p23 (as well as hsp70, although not shown here), and results in a corresponding increase in telomerase activity in P69 cells ('Q' - quantitation).

We have also over-expressed hTERT in these nontumorigenic P69 cells, which showed a corresponding and consistent increase in telomerase activity without a change in chaperone protein levels, including hsp90, p23, and hsf-1 (Figure 5), as well as hsp70 (data not shown).

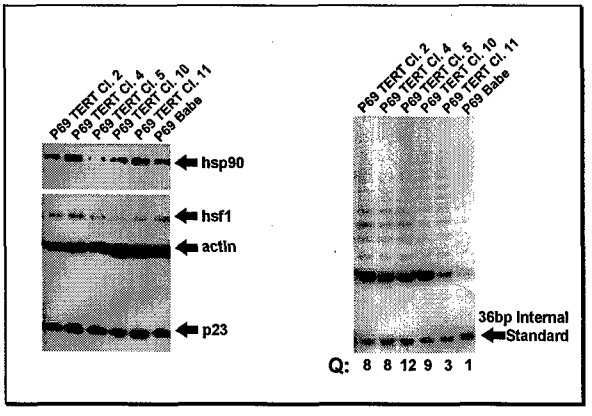

Figure 5. Exogenous hTERT expression results in increase telomerase activity. Increased telomerase activity is apparent following overexpression of hTERT in P69 cellular clones without any changes in chaperone levels (including hsp70, not shown) and the hsf-1 transcription factor.

The use of a constituitively active mutant form of the Ha-ras gene in immortal, yet nontumorigenic, cells, often results in tumorigenic transformation as assessed by anchorage independent growth in soft agar and/or tumor formation in nude mice. Therefore, to assess the functional role of chaperones and telomerase during transformation, we introduced the oncogenic form of ras into the P69 cells, which should give us a positive control for transformation as well as determine whether chaperones or telomerase were upregulated. Oncogenic ras (RasV12) expressing clones do not show a substantial increase in telomerase activity (variability is consistent with that observed for pBABEpuro clones) nor do they upregulate the hsp90, hsp70, or p23 chaperones (Figure 6 shows no change in hsp).

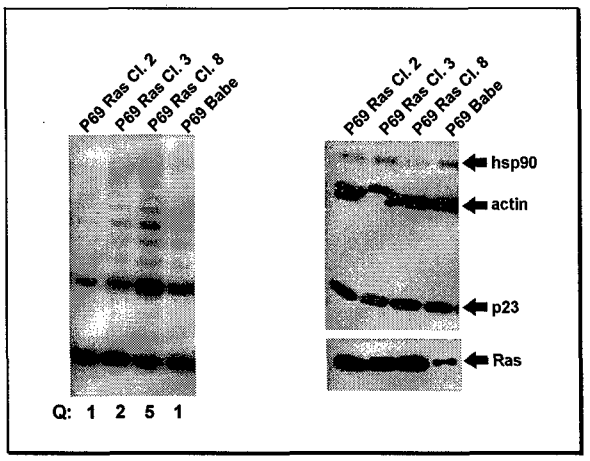

Figure 6. Othe effects of ver-expression of oncogenic ras (RasV12) in non-tumorigenic prostate cells. A substantial increase in oncogenic ras has no effect on global chaperone levels and does not result in an increase in telomerase activity.

We have tested all of the hsf-1, hTERT and ras clones for their growth rates and their ability to form colonies in soft agar, an in vitro assay to assess transforming ability of potentially tumorigenic cell lines. We find that there are minimal differences in growth rate for all of the clones when compared to either uninfected P69 cells or vector (pBABEpuro) infected clones (data not shown). We also observe a modest transforming ability with the vector controls and absolutely no colony formation with the hsf-1 clones in the soft agar assay (Figure 7). Curiously, we find a significant colony forming efficiency for the hTERT expressing P69 cells when compared to the M12 metastatic subline, suggesting that these cells could be transformed (Figure 7). However, if oncogenic ras (RasV12) is infected into P69 cells, significant colonies are found in a soft agar assay (note the difference in scale on the Y-axis for the ras clones versus the hTERT or hsf-1 studies in Figure 7). Further tests using nude mice will be accomplished for each of the hTERT, hsf-1, and oncogenic ras clones in the no-cost extension phase of the grant. Because these data are very preliminary, we do not show them here, but these cells continue to be under investigation.

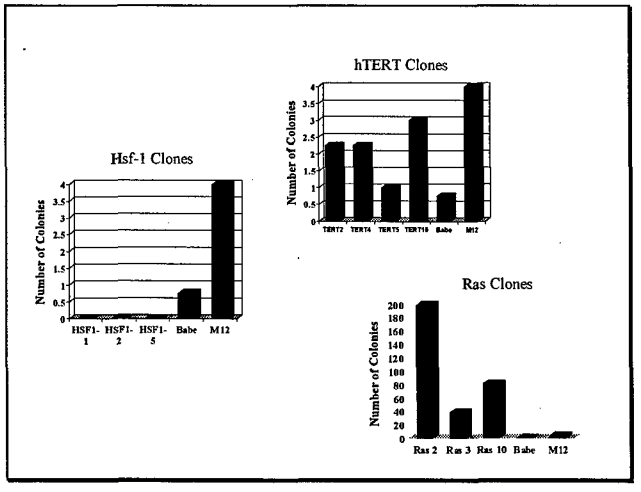

Figure 7. Soft agar analysis of hsf-1-, hTERT-, and oncogenic ras-expressing P69 clones. Individual clones were tested for their ability to grow under anchorage independent conditions. None of the hsf-1 clones formed colonies, while some of the hTERT clones formed colonies consistent with the tumorigenic M12 cells. The ras clones formed many more colonies than any of the other cell lines tested (note the scale for the ras clones).

One of our original hypotheses was that the increase in telomerase activity during progression was the result of elevated chaperone-mediated folding of the telomerase complex, rather than an increase in the expression of telomerase components (hTERT and hTR remain unchanged during

transformation) (Akalin et al., 2001). Consequently, the expectation when ectopically expressing hTERT in the non-tumorigenic P69 cells was that there would be no increase in activity without an increase in assembly as chaperones are limiting. Interestingly, we observe a significant increase in activity after hTERT expression (see Figure 5). Because endogenous hTERT is expressed at very low levels in most tumor-related cells, it is likely not a prominent target for the hsp90 chaperone complex, but when hTERT is suddenly over-expressed, it becomes more available for chaperone-mediated assembly. As such, we have subsequently shown that in hTERT over-expressing cells, as well as hsf-1 over-expressing cells with elevated telomerase activity, more of the hsp90 and p23 chaperones are observed in the nucleus of the cells, suggesting more association of these chaperones with telomerase (Figure 8).

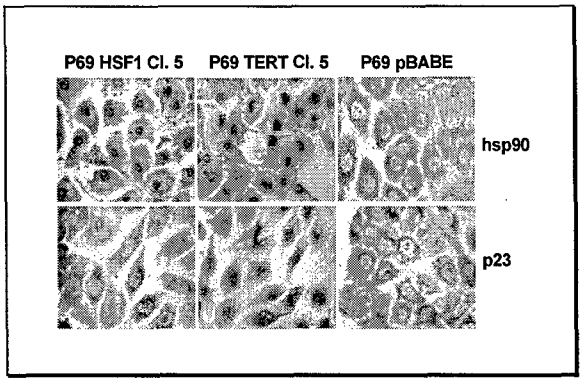

Figure 8. Ectopic expression of hTERT or hsf-1 results in increased nuclear staining of hsp90 and p23. Using antibodies specific for hsp90 and p23, P69 cells with hTERT or hsf-1 over-expressed (both have increased telomerase activity as well) exhibit elevated nuclear immunohistochemical staining when compared to the vector control cells (P69 pBABE).

We have also determined that more hsp90 is associated with hTERT in the over-expressing cells, suggesting that telomerase is a more abundant chaperone target. Figure 9 shows that more telomerase activity is precipitated with hsp90 and p23 antibodies in ectopic hTERT cells when compared to vector controls.

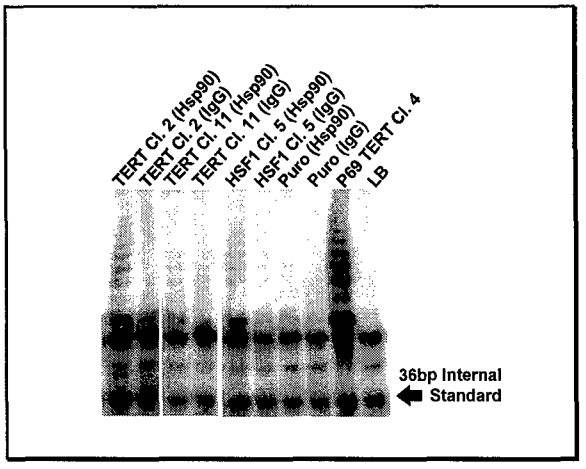

Figure 9. Increased association of telomerase and chaperones in ectopic hTERT cells. The indicated isogenic P69 cells were subjected to immunoprecipitation for cells over-expressing either hTERT or hsf-1 with an hsp90 antibody. Immunoprecipitates, assayed by TRAP analysis, indicate a greater demand for hsp90 association in the hTERT and HSF1 cell lines. P69 TERT Cl.4 serves as an input control and LB is lysis buffer (negative control).

Objective #2: **Determine the cellular and molecular consequences of targeted inhibition of chaperones and/or telomerase using pharmacological and genetic approaches in tumorigenic prostate cancer cells.**

Despite several studies describing the effect of chaperone inhibition on telomerase activity, few studies have examined the long-term consequences of Hsp90 inhibition on telomere length using either pharmacological or genetic approaches. Because we observe an increase in chaperone expression and function in prostate cancer cell lines and primary prostate tumors (Akalin et al., 2001), our goal was to determine if chaperones, specifically hsp90, were targets for inhibition of telomerase activity and reversion of the tumorigenic phenotype to a less severe, non-tumorigenic state. Thus far, we have successfully employed both the pharmacological and genetic approaches to blocking chaperones using drugs (radicicol and geldanamycin) and siRNA to block hsp90 function. Initially, we found that geldanmycin was quite toxic and resulted in a decline in telomerase as well as in cell viability, an outcome that was undesirable for an indirect method of telomerase inhibition (data not shown). As we reported last year, radicicol, which binds in the ATP-binding pocket of hsp90 in a similar manner as geldanamycin and blocks hsp90s ability to associate with p23 and other chaperone targets, was capable of blocking telomerase activity in a time-dependent manner. The molecular and cellular effects of chronic inhibition of Hsp90 function were evaluated in the M12 metastatic prostate cancer cell line. Notably, M12, like many human cancer cells, expresses high levels of Hsp90 (Akalin et al., 2001), making this cell line an ideal model in which to study chaperone inhibition. Cells were treated with pharmacological agents that bind to and disrupt the ATPase activity of Hsp90. Radicicol (RAD) is a macrocyclic antifungal antibiotic that is non-toxic to the M12 prostate cancer cell line that binds to the amino-terminal ATP binding pocket on Hsp90 and disrupt function by preventing ATP hydrolysis, an essential process required for chaperone activity. Using a chronic continuous replenishment of radicicol in the M12 cells, we found a significant decrease in telomere length with onlya transient decline in telomerase activity, and significant apoptosis (100%) after 2 months of chronic treatment (Figure 10). Long-term treatments with radicicol (out to 55 days) using the 2-day treatments provided a surprising, but somewhat expected result: sudden apoptosis at day 55. This apoptotic timing is completely reproducible (at least 5 independent experiments) and virtually 100% of the cells undergo apoptosis within the 53-55 day window.

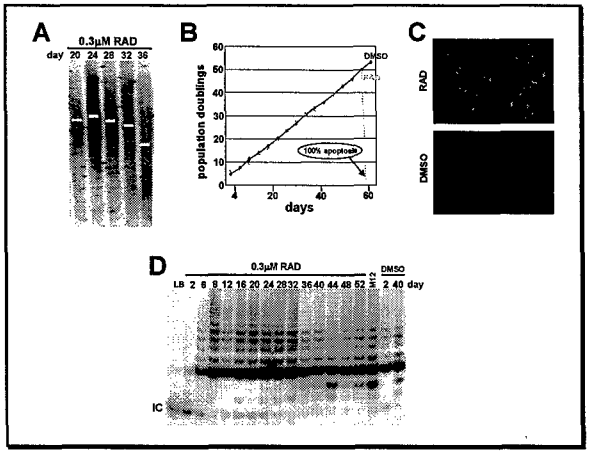

Figure 10. Telomere shortening and apoptosis following radicicol treatment of the metastatic M12 cells. Chronic treatment of M12 cells with radicicol (0.3μM) results in a telomere shortening (A), and eventually apoptosis (B & C), with only transient inhibition of telomerase activity (D). Telomeres shorten about 100bp/population doubling.

We have targeted hsp90 genetically as well with siRNA for hsp90. Figure 11 shows that although there is no decline in telomerase activity over time in the hsp90 siRNA cell clones, hsp90 is nearly undetectable in these cells, which is consistent with the transient down-regulation of telomerase in the radicicol treated cells in that there was likely an initial decline in telomerase activity after infection of the siRNA for hsp90, but this decrease was not observable as it would have occurred during selection of stable populations of cells expressing the hsp90 siRNA. Thus, we conclude that there is only a transient down-regulation of telomerase activity in prostate tumor cells treated with hsp90 inhibitors. Importantly, we find that the hsp90 siRNA clones have decreased telomere lengths (Figure 11), indicating the importance of hsp90 in the telomere maintenance process in tumor-derived cells. Nearly identical results were obtained for siRNA-mediated depletion of the p23 co-chaperone (not shown).

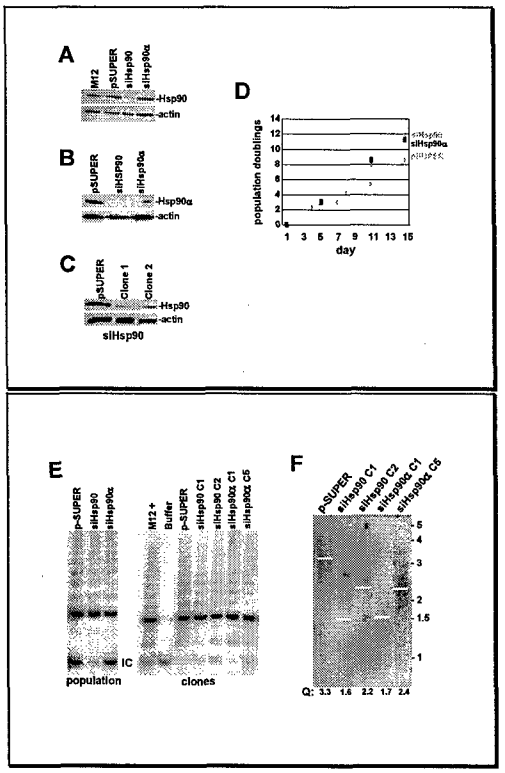

Figure 11. Genetic Inhibition of Hsp90 Causes Telomere Shortening without Altering Telomerase Activity or Cell Growth. (A) Mass cultures of M12 cells with siRNA expression specific for either Hsp90α or both Hsp90 isoforms (α and β), along with vector (pSUPER) controls, were tested for expression Hsp90 by immunoblotting. (B) Hsp90α protein levels following stable integration of the Hsp90 and Hsp90α siRNA constructs. (C) Hsp90 expression in single cell-derived clones (1 and 2) after infection with siRNA directed at both Hsp90 isoforms. (D) Growth of logarithmically cultured M12 cells following Hsp90 siRNA expression was calculated. (E) Telomerase activity was tested in M12 mass cultures (left panel) and clones (right panel) following infection with siRNA constructs directed at Hsp90. [Internal control (IC)]. (F) Telomere lengths of individual clones were analyzed Telomere-specific Southern. The average telomere length (Q) was measured densitometrically.

All of the data for inhibition of chaperones is well described in the attached manuscript, which was favorably reviewed in EMBO Journal and should be published in the next few months. Our goal was to determine why telomeres continue to shorten during RAD treatment, especially considering telomerase is functionally active. Because many different types of drug treatments have been shown to affect the production and homeostasis of free radicals, specifically the superoxide (O_2^-), we tested acutely RAD treated (at higher concentrations) and our chronically treated M12 cells for free radical production after staining with dihydroethidium (DHE) and flow cytometry. Figure 12, parts A and C, show the acute with higher concentrations of RAD and the chronic treated cells with high free radical production, which fluoresce red after staining with DHE.

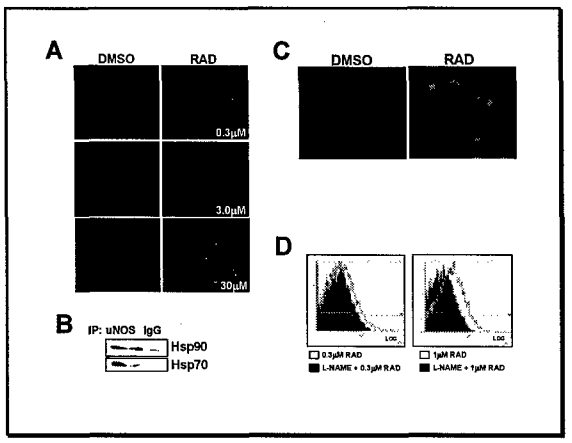

Figure 12. Radicicol Induces NOS-Dependent Free Radicals. (A) RAD induces a dose dependent increase in superoxide (O^{2-}) generation in the M12 metastatic prostate cancer cells. Cells were incubated in fresh media containing DHE for 30 minutes, followed by imaging using a fluorescent microscope. Cells with high concentrations of free radicals emit an intense red fluorescence. **(B)** Cells chronically treated with RAD or DMSO for 55 days were reseeded into 8-well chamber slides, incubated with DHE for 30 minutes, and visualized using a fluorescent microscope. Note the high concentrations of free radicals in RAD-treated cells compared to the vehicle controls (DMSO). **(C)** Cells were incubated in 0.3μM or 1μM RAD for 24hrs with or without 500μM of the NOS inhibitor L-NAME, and then incubated in fresh media containing DHE for 30 minutes. Free radical production was quantified by flow cytometry analysis and plotted on a logarithmic scale.

To determine if Hsp90 interacts with NOS in the M12 cells, we used a universal NOS antibody to co-immunoprecipitate (co-IP) NOS associated proteins and immunoblotted with antibodies specific to Hsp90 and Hsp70. Both Hsp90 and Hsp70 were associated with NOS before and after treatment with RAD (Figure 12B), consistent with the notion that chaperone inhibition does not alter Hsp90's ability to bind its target proteins. We also found that complete inhibition of the NOS pathway, using the NOS inhibitor L-NAME (500μM) to completely block production of free radicals and nitric oxide, rendering the NOS enzyme functionally inactive, causes a shift in the DHE staining intensity as quantified by flow cytometry, corresponding to a reduction in ROS production compared to RAD treatment alone (Figure 12D).

Inhibition of hsp90 with siRNA specifically knocks down hsp90 protein expression with no effect on telomerase activity in isolated clones (data not shown – see attached manuscript for a full account). Interestingly, all single cell-derived clones with hsp90 siRNA exhibited shortened telomere lengths (see attached manuscript). The similarity between pharmacological and genetic (siRNA) inhibition of Hsp90 function on growth, telomerase activity, and telomere length points to a common mechanism of action that is responsible for induction of telomere shortening in these cells. As shown with pharmacological inhibition of Hsp90, genetic knockdown of Hsp90 resulted in elevated ROS production above levels observed in cells with an empty vector (Figure 13A and 13B, right panel). Production of free radicals is reduced after a 1-hour incubation with the NOS inhibitor L-NAME (data not shown) and completely blocked after a 24-hour incubation (Figure 13B, left panel).

As both pharmacological and genetic inhibition of Hsp90 resulted in NOS-dependent free radical production, we addressed the question of whether telomere length in cells expressing siRNAs directed at Hsp90 can be maintained or even elongated if ROS production is inhibited by continuous culture with the NOS inhibitor L-NAME. Telomere length analysis in cells expressing Hsp90 siRNA constructs indicates that L-NAME allows for an increase in telomere

lengths (Figure 13C), consistent with the telomere elongation observed with both RAD and L-NAME (Figure 13C and see manuscript) and as dramatic as that observed in RAD-treated cells upon removal from the drug treatment (see attached manuscript). These data imply that a certain minimum level of Hsp90 is required by cells to maintain essential intracellular functions.

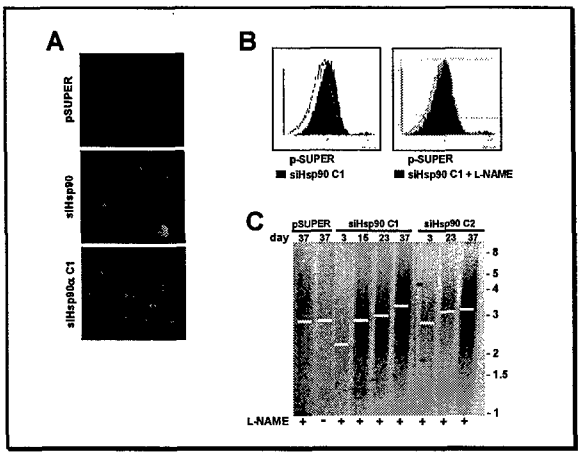

Figure 13. Inhibition of NOS-induced Free Radicals Prevents Telomere Erosion. (A) Cells expressing the vector (pSUPER) or siRNAs against total Hsp90 or Hsp90α were incubated in media containing DHE for 30min. After dye removal, stained cells were visualized using a fluorescent microscope (increased superoxide levels are visualized by an intense red fluorescence). **(B)** Hsp90 siRNA-expressing cells were incubated in fresh media (or media containing L-NAME for 24hrs) and then with media containing DHE for 30 minutes. Free radical production as a measure of dye uptake was analyzed by flow cytometry. **(C)** Telomere lengths were analyzed in cells expressing Hsp90 siRNAs and compared relative to pSUPER controls. DNA was isolated from cells after incubation with L-NAME and subjected to the TALA.

Our data supports a model in which Hsp90 regulates the production of nitric oxide ($^{\cdot}NO$) and superoxide (O^{2-}) through NOS, depending partially on the functional status of Hsp90 and its ability to uncouple NOS enzymatic activity. A consequence of Hsp90 deregulation is that NOS promotes the production of the O^{2-} free radical, causing extensive DNA damage and as our data shows, preferential targeting of telomeres (Figure 14).

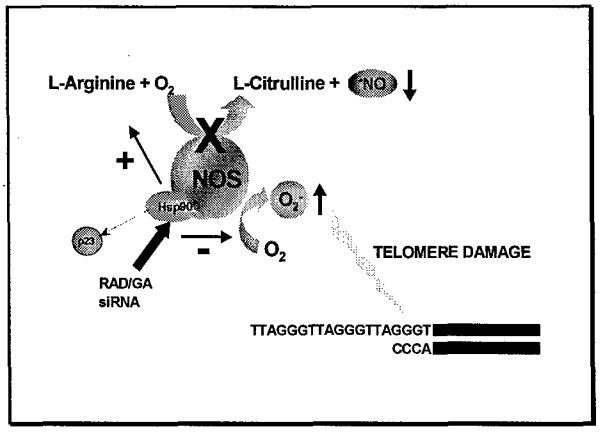

Figure 14. Model of Hsp90-Mediated Regulation of Nitric Oxide Synthase (NOS) and Free Radical-Induced Telomere Damage. NOS produces nitric oxide ($^{\cdot}NO$) as a byproduct of the reaction in which L-Arginine and oxygen are converted to L-Citrulline. Hsp90 association with NOS promotes this conversion and suppresses the pathway by which NOS generates the superoxide (O^{2-}) free radicals. Inhibition of Hsp90 function pharmacologically (RAD) or genetically (siRNA) disrupts NOS conformation to inhibit $^{\cdot}NO$ production and promote O^{2-} generation, which results in the accumulation of telomere damage (shortening), rendering the cell susceptible to apoptosis. L-NAME, by blocking both NOS pathways, protects against telomere damage and apoptosis (not depicted in the model).

We have clearly shown that blocking hsp90 function results in only a transient inhibition of telomerase activity and a significant telomere shortening in prostate tumor cells. Both pharmacologic and genetic inhibition of hsp90 results in an inability to maintain proper cellular homeostasis, causing a range of altered physiological responses including an imbalance of free radical production and telomere damage.

Key Research Accomplishments

1-establishment of chaperone-expressing P69 cells that result in an increase in telomerase activity without transforming capabilities.

2-even though reduced telomerase activity was observed to be due to limiting chaperones, expression of hTERT results in increased telomerase activity in non-tumorigenic cells, suggesting that ectopic hTERT becomes a more prominent/available target for the hsp90 chaperone complex.

3-blocking chaperone function with radicicol results in transient telomerase inhibition, telomere erosion, and eventually cell death in tumorigenic cell lines, indicating that targeting chaperones in tumorigenic prostate cancer cells may be an appropriate therapy.

4-gentetic inhibition of hsp90 or p23 using siRNA results in telomere erosion as well.

5-telomere damage in the presence of telomerase is due to the deregulation of the nitric oxide synthase pathway, which is the result of hsp90 inhibition, and an increase in free radical production.

Recommended Changes to the Proposed Work Based on Additional Findings

We have asked and received a no-cost extension to the project in order to complete the studies in Aim #1 related to tumorigenicity in nude mice with chaperone over-expression, which will complement the existing transformation data and be published within the next 12 months. We will also be publishing at least 2 additional papers on the p23 siRNA in the tumorigenic cells and the effects of over-expressing hTERT in the non-tumorigenic cells. No changes to the proposal are requested, except for the no-cost extension.

Reportable Outcomes

Manuscripts

Compton,S.A., Elmore,L.W., and Holt, S.E.. 2005Reduced Functional Hsp90 Induces a NOS-Dependent Telomere Shortening in Human Tumor Cells. Submitted, EMBO J. (favorable reviews, one additional experiment, will be sent back on May 15, 2005).

3 additional projects are near completion and will be written by the end of the summer of 2005.

Abstracts/Presentations

Elmore,L.W., C.W.Rehder, C.K.Jackson-Cook, X.Di, D.A.Gewirtz, and **S.E. Holt**. AACR: Advances in Cancer Research, Waikoloa, Hawaii. January 2004.

Compton, S.A., K.O.Jensen, L.W.Elmore, and **S.E.Holt.** AACR: Advances in Cancer Research, Waikoloa, Hawaii. January 2004.

Compton, S.A., L.W.Elmore, and **S.E.Holt.** Molecular Chaperones and the Heat Shock Response: Cold Spring Harbor Meeting, Cold Spring Harbor, NY. May 2004.

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Invited Seminars

Holt,S.E. Department of Biology, Maggie Walker Governor's School, Richmond, VA. March 2005.

Holt,S.E. Keynote Speaker, The Colorado College Biology Day, Colorado Springs, CO. April 2005.

Holt,S.E. Keynote Speaker, Pugwash Conference, Richmond, VA. March 2005.

Holt,S.E. MD/PhD Program, MCV/VCU, Richmond, VA. December 2004.

Holt,S.E. and S.A.Compton. AACR: Telomeres and telomerase in cancer. San Francisco, CA. November 2004.

Holt,S.E. Mount Desert Island Stem Cell Symposium, MDIBL, Salisbury Cove, ME. August 2004.

Development of Cell Lines

We have developed cell lines for telomerase over-expression in the non-tumorigenic prostate epithelial cell line, as well as individual P69 cell lines with oncogenic ras, hsp90, hsf-1, and p23 over-expression. We have also made the corresponding tumorigenic cell lines knocking out hsp90, p23, and telomerase using the siRNA approach. We are also using a dominant-negative approach to blocking telomerase (DN-hTERT) and hsf-1 (DN-hsf-1).

Funding Applied For

Department of Defense Breast Cancer Research Program, IDEA award, May 2003 – Awarded
National Institutes of Health (NCI), June 2003 – Declined, resubmission in July 2005
Department of Defense Breast Cancer Research Program, Predoctoral award, May 2003 – Awarded

Conclusions

Having established numerous cell lines with over-expression of chaperone-related genes, telomerase, and oncogenic ras in the non-tumorigenic P69 cell line, as well as siRNA inhibition of hsp90, p23, and telomerase, we are clearly on pace to define the cellular consequences of ectopic expression or inhibition of these proteins and their role in transformation. Our data conclusively shows that over-expression of telomerase on its own is not sufficient to elicit transformation nor does it allow for elevation of the chaperone proteins. Increased chaperone levels after hsf-1 expression has provided promising results related to stable expression and transformation, with minimal effect on tumorigenicity but significant effects on telomerase activity. We have also shown that over-expression of oncogenic ras results in tumorigenic transformation without significant effects on telomerase activity or chaperone levels. Our results for the pharmacologic and genetic inhibition of molecular chaperones and telomerase is not only interesting, but represents the first indirect method for a telomerase-independent, chaperone-mediated telomere shortening in a prostate cancer cell model system. The role of ROS and the Nitric Oxide Synthase pathway after hsp90 inhibition has been defined and the data suggest that telomere shortening is induced by free radical generation after blocking hsp90 function. Inhibition of chaperone function in tumorigenic prostate cells may represent a novel mode of prostate cancer therapy that would be useful for patients with more severe disease, which may provide a means of limiting recurrence or metastasis.

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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Shawn E. Holt, Ph.D.	Associate Professor

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.*)

INSTITUTION AND LOCATION	DEGREE (*if applicable*)	YEAR(s)	FIELD OF STUDY
The Colorado College, Colorado Springs, CO	B.A.	1985-1989	Biology
Texas A&M University, College Station, TX	Ph.D.	1989-1994	Genetics
The University of Texas Southwestern, Dallas, TX	Postdoc	1994-1998	Aging and Cancer

RESEARCH AND PROFESSIONAL EXPERIENCE:, INCLUDING GRANT SUPPORT. DO NOT EXCEED 3 PAGES.

1998-2003	Assistant Professor, Department of Pathology and Department of Human Genetics, Virginia Commonwealth University/Medical College of Virginia, Richmond, VA
2003-present	Associate Professor, Department of Pathology and Department of Human Genetics, MCV/VCU, Richmond, VA
1998-present	Member, Massey Cancer Center, Virginia Commonwealth University/Medical College of Virginia, Richmond, VA
2002-present	Adjunct Faculty, Department of Pharmacology and Toxicology, MCV/VCU, Richmond, VA
2002-present	Member, Molecular Biology and Genetics Program, MCV/VCU, Richmond, VA
2003-present	Director, Graduate Studies and Education, MCV/VCU, Richmond, VA

Awards and Honors:

2000-2003	The V Foundation Scholars Program, Cary, NC ($100,000 award)
1996-1998	NRSA Fellowship, National Institute on Aging, while at UT Southwestern, Dallas, TX
1994	Outstanding Presenter, Research Symposium, Texas A&M University, College Station, TX
1994	Outstanding Student Government Member, Texas A&M University, College Station, TX
1988-1989	Dean's List, The Colorado College, Colorado Springs, CO
1988	Most Dedicated Football Player, The Colorado College, Colorado Springs, CO
1987	Rookie of the Year, Baseball, The Colorado College, Colorado Springs, CO
1985-1987	Outstanding College Students of America

Publications (selected over the past 3+ years, from a total of 45)

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1-Busbee,D., S.Miller, M.Schroeder, V.Srivastava, B.Guntapalli, E.Merriam, **S.Holt**, V.Wilson, and R.Hart. 1995. DNA polymerase α Function and Fidelity: Dietary restriction as it affects age-related enzyme changes. International Life Sciences Institute; Risk Assesment Symposium.

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Existing/Pending Support

Active

P.I.: Shawn E. Holt, Ph.D. (20% effort)
Title: Mechanisms of Prostate Cancer Transformation
Agency: Department of Defense
Amount: $340,000 total
Duration: 12/18/01-12/17/04 (no cost extension through Dec 2005)
This project will define the role of telomeres, telomerase, and chaperones during prostate cancer progression using both pharmacological and genetic approaches.

P.I.: Shawn E. Holt, Ph.D. (20% effort)
Title: Defining the regulation of telomerase through identification of mammary-specific telomerase interacting proteins
Agency: Department of Defense Breast Cancer program (grant **# W81XWH-04-0551)**
Amount: $451,000 total
Duration: 6/1/04-5/31/07
The grant will utilize proteomic approaches to identify mammary-specific telomerase-interacting proteins as a means to define the regulation of telomerase in breast tumor cells.

P.I.: Laurence Povirk, Ph.D. (Co-I Shawn E. Holt, Ph.D., 10% effort)
Title: Tyrosyl-DNA phosphodiesterase and oxidative DNA damage
Agency: NIH
Amount: $1,687,500 total
Duration: 7/1/04-6/30/09
The major goal of this project is to determine the clarify the mechanism involving Tdp1 deficiency and define the role of oxidative stress on telomere structure and function as it relates to cancer and aging.

P.I.: Kennon R. Poynter
Mentor: Shawn E. Holt, Ph.D.
Title: Mechanisms of telomerase inhibition using small inhibitory RNAs and induction of breast tumor cell sensitization
Agency: Department of Defense Breast Cancer program
Amount: $87,000 total direct
Duration: 4/1/04-3/31/07

Pending

none

Reduced Functional Hsp90 Induces a NOS-Dependent Telomere Shortening in Human Tumor Cells

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Abstract

In most cancer cells, the lengths of telomeres, the functional DNA-protein complexes located at chromosomes ends, are maintained by the ribonucleoprotein telomerase. Hsp90 facilitates the assembly of telomerase and remains associated with the functional complex, implying a direct involvement of Hsp90 in telomere length regulation. In an effort to elucidate the effects of Hsp90 inhibition on function and viability of human prostate cancer cells, both pharmacological (radicicol) and genetic (siRNA) approaches were utilized to target Hsp90. Depletion of functional Hsp90 caused a dramatic telomere shortening followed by apoptosis. Of particular significance, these cells exhibit a high level of nitric oxide synthase (NOS)-dependent free radical production, and simultaneous treatment of cells with the NOS inhibitor L-NAME resulted in telomere elongation and prevention of apoptosis. Overall, our data suggests a novel mechanism whereby inhibition of Hsp90 disrupts free radical homeostasis and contributes directly to telomere damage, further implicating Hsp90 as a potential therapeutic target for cancer cells.

Running Title: NOS-dependent free radicals shorten telomeres

Introduction

Telomeres are complex protein-DNA structures found at the ends of vertebrate chromosomes. Telomeres act as a buffer for the gradual loss of chromosome ends that occurs as a result of incomplete DNA replication, which serves as a mitotic clock that controls cellular lifespan. Since telomeres are composed of many kilobases of non-coding repetitive sequences, these buffer sequences function as a protective mechanism to prevent the loss of genetic information stored on chromosomes. Most normal somatic cells have limited proliferative capacity and divide until critically shortened telomeres signal an irreversible growth arrest state known as cellular senescence (Hayflick, 1965; Olovnikov et. al., 1973; Watson et. al., 1972). Senescence is also dependent upon functional checkpoint machinery, including p53 and pRB, inactivation of which leads to continued growth with further telomere shortening until the second proliferative barrier known as crisis occurs (Goldstein et. al., 1990; Wright and Shay, 1992). The rare cell capable of escaping crisis always activates a telomere maintenance mechanism, which typically involves the enzyme telomerase (Counter et. al., 1992; Kim et. al., 1994).

Telomerase is an obvious chemotherapeutic target (Shay and Bacchetti, 1997). Telomerase activity requires two core components, hTERT and hTR (Feng et. al., 1995; Weinrich et. al., 1997; Masutomi et. al., 2000), to be assembled into a functionally active enzyme by the Hsp90 chaperone complex (Holt et. al., 1999). We have previously demonstrated that chaperones are essential for optimal telomerase assembly *in vitro* (Holt et. al., 1999) and that Hsp90 itself remains associated with the functional telomerase complex (Forsythe et. al., 2001).

In a human prostate cancer model, increased assembly of telomerase by chaperones, including Hsp90, has been shown to correlate with prostate cancer progression, which is defined as increased aggressiveness *in vivo* (Akalin et. al., 2001). These findings indicate that increased

expression of the Hsp90 chaperone complex with the associated activation of telomerase activity may be important steps in prostate cancer formation (Holt et. al., 1999; Akalin et. al., 2001). While telomerase in cancer progression has been widely studied (reviewed by Shay and Bacchetti, 1997), the role of chaperones in carcinogenesis and their interplay between telomerase and its substrate, the telomere, are less well defined.

Many studies indicate that Hsp90 chaperone inhibitors, such as geldanamycin (GA), 17-allylamino-17-demethoxy-geldanamycin (17-AAG), and Radicicol (RAD), may be clinically useful as therapeutic agents for cancer patients (reviewed by Neckers, 2002; Goetz et. al., 2003). These inhibitors are capable of simultaneously targeting multiple Hsp90-associated proteins that are important in tumorigenicity, including N-ras, Ki-ras, HER-2, c-Raf-1, Akt, and mutant p53, ultimately resulting in the induction of cytostasis and/or apoptosis in cancer cells (Solit et. al., 2003; Kim et. al., 2003; Hostein et. al., 2001). Hsp90 is also involved in the production of free radicals from the nitric oxide synthase (NOS) pathway (Masters et. al., 1996; Porasuphatana et. al., 2002; Ou et. al., 2003; Ou et. al., 2004). Despite several studies describing the effect of chaperone inhibition on telomerase activity, few studies have examined the long-term consequences of Hsp90 inhibition on telomere length using either pharmacological or genetic approaches. Thus, the goal of our study was to establish a direct link between NOS-induced free radical production and telomere damage after genetic and/or pharmacologic disruption of Hsp90 function.

Results

Telomere Erosion after Pharmacologic Inhibition of Hsp90

The molecular and cellular effects of chronic inhibition of Hsp90 function were evaluated in the M12 metastatic prostate cancer cell line (Bae et al., 1994; Bae et al., 1998). Notably, M12, like many human cancer cells, expresses high levels of Hsp90 (Akalin et al., 2001), making this cell line an ideal model in which to study chaperone inhibition. Cells were treated with pharmacological agents that bind to and disrupt the ATPase activity of Hsp90. Geldanamcyin (GA) and its analog 17-allylamino-17-demethoxy-geldanamycin (17-AAG) are benzoquinone ansamycin antibiotics that are commonly used to study Hsp90 function and are currently in clinical trials as therapeutic cancer drugs (Neckers, 2002). A second class of Hsp90 inhibitor, Radicicol (RAD), is a macrocyclic antifungal antibiotic that appears less toxic to the M12 prostate cancer cell line and was better suited for chronic Hsp90 inhibition studies. All of these pharmacological agents bind to the same amino-terminal ATP binding pocket on Hsp90 and disrupt function by preventing ATP hydrolysis, which is an essential process required for chaperone activity.

Traditional studies using Hsp90 inhibitors utilize an acute treatment, assessing the cellular effects in the short-term (Neckers, 2002). To determine the long-term consequences of Hsp90 inhibition after chronic exposure to the less toxic RAD compound, M12 prostate cancer cells were cultured in media containing a low concentration of RAD for 60 days. DNA was isolated periodically from vehicle- (DMSO) and RAD-treated M12 cells, and telomere lengths were analyzed. We find that telomeres remain at a constant level ranging between 3.5-4.0kb in the presence of vehicle, while cells treated with RAD undergo a gradual telomere shortening starting at ~20 days, continuing throughout the course of treatment (Figure 1A). Average

telomere lengths quantified from multiple assays indicate that telomeres shorten from ~3.75kb to ~1.5kb during chronic RAD treatment, equating to a shortening rate of ~200bp per population doubling. Although chronic treatment of M12 cells at 0.3μM RAD could be achieved without effects on cell viability/toxicity in the short-term, continued treatment resulted in a delayed induction of cell death after ~55 days (n=6; death reproducibly occurred between days 54-60) (Figure 1B). We found that nearly 100% of the treated cells undergo apoptosis as assessed by TUNEL (Figure 1C). Cells treated with vehicle are TUNEL-negative (Figure 1C), suggesting that critically short telomeres in chronically treated cells signal the activation of the apoptotic cascade.

To characterize the mechanism underlying the induction of telomere erosion and delayed apoptosis, we analyzed the effects of chronic RAD treatment on telomerase activity. RAD treatment reduced telomerase activity as expected, but telomerase inhibition was only transient, and after one week, activity was restored (Figure 1D). Furthermore, the level of telomerase activity detected after 8 days of RAD treatment was comparable, if not higher than in untreated M12 cells or cells treated with vehicle. In fact, all three Hsp90 inhibitors were capable of transiently inhibiting telomerase activity at concentrations that did not affect cell viability (data not shown). Together, these data suggest a mechanism of telomere erosion independent of telomerase inhibition.

One possible explanation for the lack of chronic telomerase inhibition is that Hsp90 function is reestablished during chronic RAD treatment. Inhibition of Hsp90 results in the ubiquitination of a number of its bound target proteins, which are then targeted to the ubiquitin-mediated proteasome degradation pathway (Neckers, 2002). To determine if the cellular response to RAD remains constant throughout the course of treatment, protein samples were

collected at various time-points and analyzed for global changes in the levels of ubiquitinated proteins. As expected, chronic RAD treatment increases the levels of ubiquitinated proteins compared to cells treated with vehicle (Figure 1E) and those levels remain constant throughout the course of treatment, indicating that normal Hsp90 function remains inhibited in RAD-treated cells.

Free Radical Production in Cells Chronically Treated with Radicicol

The observation that telomeres shorten in the presence of detectable telomerase activity may at first appear contradictory to our understanding of how telomerase maintains telomeres. We therefore set out to establish the mechanism of telomere shortening in RAD-treated cells. Interestingly, inhibition of Hsp90 by GA, 17-AAG, and RAD have been implicated in the production of oxidative free radicals by disruption of the nitric oxide synthase (NOS) pathway (Song et. al., 2002; Ou et. al., 2003; Ou et. al., 2004). Therefore, we asked whether RAD treatment might induce oxidative free radicals that contribute to the telomere shortening.

M12 cells were exposed to various concentrations of RAD for 24 hours, washed free of excess drug and incubated with 5μM dihydroethidium (DHE), a reduced form of ethidium dye that is oxidized by reactive oxygen species (ROS) to fluorescent ethidium, which incorporates into DNA. Cells treated with increasing concentrations of RAD have a concentration-dependent increase in ROS levels as determined by DHE staining (Figure 2A). Based on this finding, it is entirely possible that chronic treatment of RAD could lead to an accumulation of DNA damage induced by a constant exposure to oxidative free radicals. We then confirmed that chronic Hsp90 inhibition caused an increase ROS generation by demonstrating that cells treated chronically with RAD (~55 days) exhibit high levels of ROS (Figure 2B), which were clearly more intense

DNA staining than in the short-term experiments (Figure 2A). This phenomenon was further confirmed using an alternative dye that detects ROS accumulation in mitochondria (Mitotracker red) (data not shown).

To determine if Hsp90 interacts with NOS in the M12 cells, we used a universal NOS antibody to co-immunoprecipitate (co-IP) NOS associated proteins and immunoblotted with antibodies specific to Hsp90 and Hsp70. Both Hsp90 and Hsp70 were associated with NOS before and after treatment with RAD (data not shown), consistent with the notion that chaperone inhibition does not alter Hsp90's ability to bind its target proteins.

Production of Free Radicals and Telomere Shortening are Dependent on Nitric oxide Synthase Activity

Previous studies have demonstrated that the production of free radicals by Hsp90 inhibitors originate from the uncoupling of NOS enzymatic function (Ou et. al., 2003; Ou et. al., 2004). To demonstrate that free radical production in our system was derived from uncoupling of NOS, cells were incubated with 1.0μM or 0.3μM RAD for 24 hours, followed by 1 hour with the NOS inhibitor L-NAME (500μM). L-NAME completely inhibits production of free radicals and nitric oxide, rendering the NOS enzyme functionally inactive. Simultaneous incubation of RAD with the NOS inhibitor L-NAME caused a shift in the DHE staining intensity as quantified by flow cytometry, corresponding to a reduction in ROS production compared to RAD treatment alone (Figure 2C and data not shown).

To determine whether the cells (and their telomere lengths) could recover from chronic RAD treatment, cells were cultured for 49 days with 0.3μM RAD, and on day 50, drug was removed (-RAD) or treatment continued (+RAD). While the cells maintained in RAD

consistently underwent apoptosis between day 55 and 60, as shown in Figure 1C, cells removed from RAD continued to proliferate for more than 35 doublings beyond this point (Figure 3A), with growth rates that were not appreciably different from DMSO-treated cells. Thus, removing cells from RAD treatment appears to prevent further free radical production, protecting cells from telomere damage and induction of cell death.

We further hypothesized that telomere lengths were maintained or elongated in the absence of drug, and that telomerase would be capable of restoring telomere length after RAD removal. Again, telomere lengths of DMSO-treated cells (Figure 3B, lane 1 and 2) were found to be significantly longer than those cells maintained in RAD (Figure 3B, lane 3). However, continued culture of cells with shortened telomeres without RAD resulted in gradual telomere lengthening, suggesting that telomerase is more efficient at maintaining telomeres in the absence of free radical generation. Furthermore, this telomere elongation protected cells from RAD-induced apoptosis, indicating that critically short telomeres contribute to drug-induced apoptosis.

To determine if inhibition of NOS would also prevent telomere shortening and delayed apoptosis in RAD-treated cells, cells were treated chronically for 44 days, and on day 45, cells were maintained in RAD (or DMSO) in the continuous presence or absence of the NOS inhibitor L-NAME. Cells treated with RAD alone died as expected, while cells co-cultured with L-NAME continued to proliferate well beyond the point at which RAD-treated cells underwent delayed apoptosis (Figure 4A). Telomere lengths in control cells (DMSO, or DMSO and L-NAME) were similar to lengths described for untreated cells. While chronic RAD treatment resulted in very short telomeres consistent with the data described above, telomere lengths of cells treated with the combination of RAD and the L-NAME NOS inhibitor appeared to increase (Figure 4B). This observation that simultaneous treatment with RAD and NOS inhibitors prevents free radical

production and cell death directly implicates NOS as the source of the telomere damage. Thus, it appears that without constant damage of telomeres by free radicals, a functional telomerase is capable of gradually elongating telomere lengths, or at least maintaining telomeres at short lengths, thereby preventing apoptosis.

Genetic Inhibition of Hsp90 Function

Our findings are consistent with other reports demonstrating that GA, 17-AAG, and RAD can uncouple NOS enzymatic activity, which results in the increased production of superoxide (Pritchard et. al., 2001; Ou et. al., 2003; Ou et. al., 2004). The novel finding that Hsp90 inhibition results in the gradual loss of telomere length and eventually delayed apoptosis suggests that an accumulation of free radical-induced telomere damage may occur over time. Because of concerns of drug specificity and potential RAD/L-NAME interactions, we chose to address the mechanism of telomere erosion and free radical homeostasis genetically by creating stable cell lines expressing siRNA hairpin constructs that specifically target Hsp90 mRNA.

Hsp90 exists in two isoforms encoded on separate genes referred to as HSP84 and HSP86 in murine cells (Moore et. al., 1990) and Hsp90α and β in human cells (Rebbe et. al., 1989). In humans, Hsp90β is constitutively expressed and moderately inducible; whereas, Hsp90α is generally expressed under low basal levels that are induced dramatically in response to stress (Goetz et. al., 2003). For Hsp90 siRNA constructs, target cleavage sequences were selected in a region that is conserved in all members of the Hsp90 family. In addition, a similar siRNA was created to specifically target only the Hsp90α isoform. While there is evidence that suggests there is some overlap in function (Picard et. al., 1990; Xu and Linquist, 1993; Cox and Miller, 2003), there are also some Hsp90 isoform-specific functions as well (Eustace et. al., 2004).

M12 prostate cancer cells were infected with vector (pSUPER), the Hsp90 siRNA (targets both Hsp90 α and β), or the Hsp90α siRNA, and expression of Hsp90 and Hsp90α were monitored by immunoblotting. Compared to empty vector controls, those cells stably expressing the α/β Hsp90 siRNA have a considerable reduction of Hsp90 protein expression (Figure 5A), while cells expressing the Hsp90α siRNA show only a reduction in Hsp90α (Figure 5B). Single cell-derived clones were isolated, and two clones that had at least a ~50% reduction in Hsp90 protein abundance were used in the remainder of these studies (Figure 5C). As we were unable to isolate individual clones with much less than 50% reduction in Hsp90 expression, this is presumably the minimum level of Hsp90 chaperone activity required to maintain cell survival.

Populations of cells infected with Hsp90α constructs did not appear to have an observable change in total Hsp90 (Hsp90 α and β) chaperone expression level (Figure 5A). However, the expression of the Hsp90α isoform was dramatically reduced (Figure 5B). This observation is not unexpected since a Western blot for total Hsp90, which makes up 2-4% of total cellular protein, small changes in the weakly expressed Hsp90α isoform can be masked (Goetz et. al., 2003).

Cells stably infected with siRNA constructs targeting Hsp90 experienced no notable changes in proliferation, indicating that cells can survive with reduced expression of these chaperones without any observable cellular toxicity (Figure 5D). Furthermore, there were no measurable changes in telomerase activity between samples that expressed siRNA inserts and empty vectors controls (Figure 5E). Analysis of telomerase activity from individual clones infected with the different siRNA constructs also show minimal changes in telomerase activity (Figure 5E). Creation of cell lines that stably express siRNA-like constructs requires a 5-7 day antibiotic selection process, followed by analysis by immunoblotting or TRAP. Therefore, it is

likely that a transient inhibition of telomerase activity, like that observed in drug-treated cells between days 2-6, would be missed in siRNA cells due to time-frame of the selection process. Taken together, the data from both the drug and genetic inhibition of Hsp90 studies described above leads to the conclusion that there are no long-term effects on telomerase activity in the presence of reduced functional Hsp90.

Telomere length analysis confirmed the findings using RAD, indicating that reduction in Hsp90 function, whether global Hsp90 or Hsp90α, leads to telomere shortening (Figure 5F). The extent of shortening was similar in both Hsp90 siRNA infected cells and chronic RAD-treated cells with a reduction from 3.3kb down to 1.5-2kb (Figure 5F).

NOS-dependent Free Radicals Production Induces Telomere Shortening after Genetic Inhibition of Hsp90

The similarity between pharmacological and genetic (siRNA) inhibition of Hsp90 function on growth, telomerase activity, and telomere length points to a common mechanism of action that is responsible for induction of telomere shortening in these cells. Given that we observed increased NOS-dependent free radical production combined with telomere shortening following chronic RAD exposure, cells were similarly evaluated following genetic inhibition of Hsp90 for the production of free radicals. As shown with pharmacological inhibition of Hsp90, genetic knockdown of Hsp90 resulted in elevated ROS production above levels observed in cells with an empty vector (Figure 6A and 6B, right panel). Production of free radicals is reduced after a 1-hour incubation with the NOS inhibitor L-NAME (data not shown) and completely blocked after a 24-hour incubation (Figure 6B, left panel).

As both pharmacological and genetic inhibition of Hsp90 resulted in NOS-dependent free

radical production, we addressed the question of whether telomere length in cells expressing siRNAs directed at Hsp90 can be maintained or even elongated if ROS production is inhibited by continuous culture with the NOS inhibitor L-NAME. Telomere length analysis in cells expressing Hsp90 siRNA constructs indicates that L-NAME allows for an increase in telomere lengths (Figure 6C), consistent with the telomere elongation observed with both RAD and L-NAME (see Figure 4B) and as dramatic as that observed in RAD-treated cells upon removal from the drug treatment (see Figure 3B). These data imply that a certain minimum level of Hsp90 is required by cells to maintain essential intracellular functions. Inhibition of Hsp90 results in an inability to maintain proper cellular homeostasis, causing a range of altered physiological responses including an imbalance of free radical production and telomere damage.

Discussion

Our data supports a model in which Hsp90 regulates the production of nitric oxide ($^{\bullet}NO$) and superoxide (O^{2-}) through NOS, depending partially on the functional status of Hsp90 and its ability to uncouple NOS enzymatic activity (Ou et. al., 2002; Ou et. al., 2004). A consequence of Hsp90 deregulation is that NOS promotes the production of the O^{2-} free radical, causing extensive DNA damage and as our data shows, preferential targeting of telomeres (Figure 7).

Hsp90 Inhibitors Indirectly Induce Telomere Shortening and Transiently Inhibit Telomerase. Several reports have described the use of direct telomerase inhibition as potential adjuvants to traditional cancer therapies and to prevent metastasis and recurrence of residual disease after traditional forms of cancer treatment (Herbert et. al., 1999: Shammas et. al., 1999; Hahn et. al., 1999). Our goal was to determine if Hsp90 inhibition could be used to indirectly target telomerase, which is particularly appealing because there are several established antibiotic inhibitors of Hsp90 that are well tolerated in humans and are currently in clinical trials as potential cancer therapies (Neckers, 2002; Kelland et. al., 1999). Although there were only limited and transient effects on telomerase activity, our results are consistent with those of others describing indirect targeting of telomerase in colon adenocarcinoma and melanoma cell lines using benzoquinone antibiotics (Villa et. al., 2003 and Hostein et. al., 2001). Here, we show that a functionally related but different class of antibiotic, Radicicol, was also capable of transiently inhibiting telomerase at low doses without detrimental effects on viability but that chronic inhibition of Hsp90 results in telomere shortening and ultimately in cell death through the generation of reactive oxygen via deregulation of the NOS pathway.

Previous studies with melanoma cells only showed telomerase inhibition after 3 days, yet

had no measurable effects on telomere lengths after chronic (21 days) benzoquinone treatment (Villa et. al., 2003). Based on our results, we predict that telomerase was reactivated in these melanoma cells after the first 72 hours of chronic benzoquinone treatment and that the reestablished telomerase activity protected cells from telomere shortening. Furthermore, telomeres in these melanoma cells are substantially longer (~6-15kb) compared to those in the prostate cancer cells (~3.75kb) used in this study. Thus, the previously used melanoma cells are suboptimal for monitoring short-term telomere erosion, since long telomeres may mask any gradual length changes during drug exposure.

It is interesting that telomerase activity recovers in cells during chronic Hsp90 inhibition, suggesting telomerase assembly in absence of Hsp90 function. It may be that Hsp90 is not strictly required for assembly of extractable telomerase but necessary for telomere elongation, consistent with our previous findings that Hsp90 is associated with functional telomerase (Forsythe et al., 2001). Alternatively, and perhaps more simply, because telomerase is a low abundant Hsp90 target, it may also be that there is enough residual functional Hsp90 in cells to fully assemble active telomerase.

Inhibition of Hsp90 Disrupts Free Radical Homeostasis. Increasing evidence indicates that Hsp90 is intimately involved in the production of oxidative damage as a result of its interaction with NOS. Hsp90 functionally associates with NOS, an enzyme involved in converting L-arginine into L-citrulline and nitric oxide ($^{\bullet}NO$) (Masters et. al., 1996). These studies demonstrate that NOS, in addition to producing $^{\bullet}NO$, is also capable of producing O^{2-} and H_2O_2 free radicals (Porasuphatana et. al., 2002). In particular, inhibitors of Hsp90, including GA, 17-AAG, and RAD, can disrupt NOS activity, resulting in an increase in NOS-dependent O^{2-}

radicals (Ou et. al., 2003; Ou et. al., 2004). Our data shows a concentration-dependent increase in free radical production after acute treatment of human tumor cells with the Hsp90 inhibitor RAD. Similarly, genetic inhibition of Hsp90 using a specific siRNA promoted free radical generation. We further demonstrate that cells with deregulated Hsp90 accumulate elevated free radical damage and that a specific NOS inhibitor (L-NAME) blocks ROS production. Taken together, these results clearly demonstrate a direct link between DNA damage induced by inhibition of Hsp90 and deregulation of the NOS pathway.

NOS-induced Free Radical Production Directly Damages Telomeres. It is well established that oxidative free radicals can damage DNA, including telomeric DNA. When combined with the finding that RAD induces high levels of ROS, these two observations suggest that the increased production of free radicals by Hsp90 inhibition may contribute to telomerase-independent telomere damage. Furthermore, several lines of evidence suggest that telomeres may act as preferential targets for free radical damage. When telomeric oligonucleotides were exposed to oxidative stress using H_2O_2 and O^{2-}, DNA damage (i.e. cleavage sites and adducts) preferentially occurred at the 5' site of 5'-GGG-3' sequence in the telomere oligonucleotide (Kawanishi et. al., 1999). In accordance with these findings, Fenton reactions (between H_2O_2 and Fe^{2+}) cause preferential cleavage sites located at the 5' end of the sequence RGGG in a plasmid containing 81-telomere repeats, along with a 7-fold increase in strand breaks compared to controls with the same guanine content (Henle et. al., 1999). Henle et al. propose that telomeric DNA may protect the genome from DNA damage by attracting oxidative damage to non-coding telomeric sequences, which seems plausible given the telomere's repetitive, G-rich sequence and its overall structure. These studies together with our current data allow us to predict that the

extent of telomeric damage induced by a lack of functional chaperones is greater than the compensatory mechanisms of telomerase, since detectable telomerase activity failed to protect against telomere shortening in cells with inhibited Hsp90. Interestingly, prostate cells are capable of recovering from RAD even after extensive telomere damage as withdrawal from RAD restored telomere lengths to levels observed in untreated cells. This, in turn, suppresses the induction of apoptosis in these cells, thus demonstrating a connection between critically short telomeres and induction of apoptosis.

Further support for this model was demonstrated in our system by showing that the NOS inhibitor L-NAME was capable of eliminating free radical production and protecting cells from telomere shortening and apoptosis, directly implicating the source of the telomere damage as NOS-induced free radicals. While inhibition of NOS did not lead to complete recovery of telomere lengths when simultaneously cultured with RAD, telomere lengths were maintained at an adequate length to continue normal proliferation and prevent apoptosis. We also provide additional evidence that genetic Hsp90 inhibition leads to telomere shortening and that these cells experience more than a 50% reduction in Hsp90 when compared to empty vector controls. The fact that telomerase is expressed in both RAD- and siRNA-treated cells further suggests that this is a telomerase-independent telomere shortening mechanism. In addition, the fact that both conditions resulted in high levels of NOS-dependent free radical production provides strong convincing evidence that these two processes are directly linked.

Free radicals have been demonstrated to be intimately involved in the induction of apoptosis, yet the mechanism(s) of free radical-induced apoptosis in response to different stimuli is poorly understood. While both free radicals and telomere shortening can induce apoptosis, we do not understand how these biologic processes interconnect. Consistent with our findings, an

earlier study has demonstrated that the consequence of •OH radical-induced apoptosis was telomere erosion (Ren et. al., 2001), indicating a strong correlation between free radical-induced telomere shortening and apoptosis and adding additional support to our model.

Tumor Selectivity of Hsp90 Inhibitors. Free radicals are produced in all cells as natural byproducts of cellular reactions such as respiration and are known to cause damage to DNA including telomere damage (von Zglinicki et. al., 1995; von Zglinicki et. al., 2000; von Zglinicki et. al., 2002). Normally, cells buffer the production of these damaging free radicals with scavenging enzymes, including superoxide dismutase (SOD) and peroxides that convert free radicals to inactive products (Halliwell et. al., 1999). There is some evidence that cancer cells, due to their high rates of proliferation and metabolism, produce higher levels of free radicals compared to non-cancerous cells and that scavenging enzymes may be working at a near optimal capacity, making cancer cells more susceptible to free radical damage (Hileman et. al., 2004). In addition, Hsp90 is predominantly in an uncomplexed state in normal cells; whereas, in cancer cells, Hsp90 is complexed with other chaperones and/or targets in a highly active state (Kamal et. al., 2003). These Hsp90 complexes have been demonstrated to have a several-fold higher affinity for Hsp90 inhibitors than the uncomplexed form found in normal cells, suggesting a basis for a tumor selectivity mechanism of Hsp90 inhibitory agents (Kamal et. al., 2003). Taken together, normal human cells would likely be less sensitive to Hsp90 inhibitors than cancer cells, making such agents relevant for testing as potential anti-cancer adjuvant therapies.

Summary

Understanding the complex nature of DNA damage and apoptosis induced by Hsp90 inhibition,

both genetically and pharmacologically, will contribute to improved use of Hsp90 inhibitory compounds therapeutically and may help to identify additional agents that are likely to work synergistically to increase specific killing of cancer cells. The work presented here has provided important insights into the consequences of Hsp90 inhibition on telomerase and telomere biology. While we demonstrate telomerase inhibition in prostate cancer cells by chaperone inhibitors in the short-term, our data clearly indicate that telomerase inhibition cannot be maintained long-term using these compounds, thus revealing that anti-Hsp90 compounds would be ineffective telomerase inhibitors. However, other aspects of Hsp90 inhibition may make these agents potentially useful as adjuvant therapies. Some Hsp90 inhibitors are currently in clinical trials and have been shown to simultaneously target multiple signaling pathways involved in promoting tumorigenicity. Here, we provide additional insights into the complex pharmacology of these agents with respect to the Hsp90 inhibitor RAD. We demonstrate that RAD is capable of producing free radicals even at low concentrations, which in turn, translates into specific free radical-induced telomere damage. Moreover, we find that long-term culture of cells with the Hsp90 inhibitor RAD results in delayed apoptosis as a result of critically short telomeres. Our knowledge of how different chemotherapeutic agents may induce DNA damage and apoptosis by free radical mechanisms may potentially lead to better treatment strategies and adjuvant therapies that can prevent cancer development and disease recurrence.

Materials and Methods

Materials

Radicicol, Geladanamycin, L Nitro-Arginine Methyl Emide (L-NAME), and DMSO were purchased from Sigma. 17-AAG was kindly provided by Dr. Neal Rosen (Memorial Sloan Kettering Cancer Center, NY).

Cell Lines and Isolation of Subclones

All tumor cells were cultured in RPMI 1640 containing 5% fetal bovine serum (FBS) and supplemented with ITS (Insulin 5μg/ml, Transferring 5μg/ml and Selenium 5ng/ml, Collaborative Research), dexamethasone (0.1μM) and gentamicin (0.05mg/ml). The human prostate epithelial cell line, M12, used in these studies has been extensively characterized (Bae et. al., 1994; Bae et. al., 1998; Akalin et. al., 2001). All cells were mycoplasma free, as assessed by the mycoplasma T.C. Rapid Detection System (Gen-Probe, San Diego, CA). Isolation of subclones was achieved by seeding 500 cells onto a $15cm^2$ tissue culture plate and then expanding individual colonies after 2 weeks.

Design of siRNAs

siRNA sequences were designed according to the manufacturer's recommendations for use with the pSUPER.retro (SUPpression of Endogenous RNA) system (Oligoengine). Briefly, a 19-nucleotide target sequence specific to the chaperone of interest was identified using Dharmacon siDESIGN center and criteria reviewed as described (Brummelkamp et. al, 2002). Candidate sequences were used to synthesize a pair of 64-mer oligonucleotides with the target sequence in sense and anti-sense orientation separated by a 6-nucleotide spacer region (to allow hairpin

formation). Linkers containing 5' Bgl II and 3' Hind III sites allowed the directional cloning of the annealed oligonucleotides into the pSUPER.retro. The complementary oligonucleotides were annealed according to the manufacturer's instructions prior to cloning into the pSUPER.retro vector. The 64-mer siRNA sequences were synthesized for both isoforms of Hsp90 (α and β) and the α isoform only, as follows: **HSP90 (α and β): 5'**-GATCCCCGTTT GAGAACCTCT GCAAATTCAA GAGATTTGCA GAGGTTCTCA AACTTTTTGG AAA-**3'** and **HSP90α: 5'**-AGCTTTTCCA AAAAGTTTGA GAACCTCTGC AAATCTCTTG AATTTGCAGA GGTTCTCAAA CGGG-**3'**. Prior to transfecting cells with the vectors containing the designed oligonucleotide sequences, the presence of the correct insert was confirmed by sequencing using the primers provided with p-SUPER.retro at positions 1242-1257 and its complement, 2645-2629.

Generation of Retroviral Cell Lines and Infection

The pSUPER.retro vectors were transfected using Fugene Reagent (Roche) into the Phoenix A competent cell line, as recommended by the manufacturer. The resulting retroviral supernatant was collected at 6hrs and 24hrs, filtered through a 0.45μM filter, and incubated with M12 cells for 6hrs. Cells were allowed 24hrs to recover before initiation of 10μg/ml puromycin selection, followed by isolation of individual clones as above.

Radicicol (RAD) Treatments

For chronic RAD studies, M12 cells were seeded at 25% confluency and exposed to freshly prepared 0.3μM RAD (1mM stock in DMSO) or an equivalent volume of DMSO in standard RPMI culture media. RAD- (or vehicle-) containing media was replaced every 2 days. Cells were

cultured and reseeded at 25% confluency, usually on the fourth day. At each passage, total cell numbers were determined, and population doublings were calculated using the formula [$\log_{10}$(number of cells counted/number of cells plated)]/0.3. All cells were kept in log phase growth during the calculation of proliferation rates.

TdT-Mediated d-UTP-X Nick End Labeling (TUNEL) Assay

Cells were fixed directly on a 6-well chamber slide in 4% Formaldehyde for 10 minutes. Next, the cells were washed twice in PBS for 5 minutes each and fixed in acetic acid:ethanol (2:1) at –20°C for 5 minutes. Slides were washed, blocked in 1mg/ml BSA in PBS for 30 minutes at room temperature, and incubated with an enzyme mix containing 4µl terminal transferase, 5X reaction Buffer, 25mM $CoCl_2$ and Fluorescein 12-dUTP (Boehinger Mannheim) for 60 minutes at 37°C in a humidified chamber under light sensitive conditions. Cells were washed twice in PBS for 5 minutes, mounted in Vectashield (Vector Labs), and stored at 4°C. Representative images were captured using an OLYMPUS IX70 fluorescent microscope (Optical Elements Corporation).

Immunoblotting

Cells grown in DMSO, radicicol (0.3µM), or after stable integration of siRNAs were harvested at ~80% confluency, washed in PBS, and lysed for 30 minutes on ice in RIPA Buffer (50mM Tris pH 7.4, 150mM NaCl, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS, 1% Aprotinin and 100mM DTT). For immunoblotting, total cellular protein (25-50µg) was denatured at 95°C for 10 minutes and subjected to 12% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (BioRad), and the membrane was blocked using 5% nonfat dried milk. Nitrocellulose membranes were incubated with anti-Hsp90, anti-p23, anti-actin (all 1:5000 dilution) (Akalin et.

al., 2001), anti-ubiquitin (Stressgen, Vancouver, BC), or anti-uNOS (Stressgen) (1:1000), followed by incubation with mouse or rabbit secondary antibody conjugated with horseradish peroxidase (1:5000) (BioRad). Detection involved the use of Pierce ECL kit with exposure to Kodak X-OMAT film. Semi-quantification of bands was achieved by calculation of the signal from the protein of interest relative to β-actin using spot densitometry software (ChemiImager 4400, Alpha Innotech Corporation).

Measurements of Superoxide Generation

Two different dyes were utilized to assess the production of free radicals: dihydroethidium (DHE) (Sigma) is a cell permeable dye that is oxidized to fluorescent ethidium bromide by superoxides and intercalates into DNA, and Mitotracker Red (Molecular Probes) is also oxidized by endogenous superoxides but accumulates in mitochondria as a fluorescent product. After treatment, cells were transferred onto 6-well chamber slides at 50-80% confluency. Prior to addition of DHE, cells were thoroughly rinsed to remove all traces of drug and then incubated in fresh media containing 0.5μM DHE or MTR for 30 minutes at 37°C in the dark. Intact cells were washed to remove excess dye, and images were captured by fluorescent microscopy under identical acquisition settings. Alternatively, cells were treated as above except after incubation in dye, cells were trypsinized, washed, and resuspended in PBS at $1x10^6$/ml for analysis using flow cytometry.

Telomeic Repeat Amplification Protocol (TRAP Assay)

Telomerase activity was measured according to the manufacturer's instructions using the TRAP-eze detection kit (Serologicals, Purchase, NY), as previously described (Akalin et. al., 2001;

Forsythe et. al., 2001). Relative telomerase activity was quantitated by ImageQuant software (Molecular Dynamics) analysis of the ratio of the telomerase ladder to the included 36bp internal standard.

Telomere Amount and Length Assay (TALA)

A telomere amount and length assay was modified from Gan and colleagues (2001) (Gan et. al., 2001). DNA was isolated from subconfluent cells using the Blood and Cell Culture DNA Isolation kit (Qiagen). Total DNA (25μg) was digested using six restriction enzymes Alu I, Msp I, Rsa I, Cfo I, Hae III, and Hinf I (Gibco-BRL) for 2 hours at 37°C. A telomere-specific probe, $(TTAGGG)_4$, was end-labeled with $^{32}P[\gamma\text{-ATP}]$. Unincorporated nucleotides were removed from the reaction using the Qiaquick nucleotide removal kit (Qiagen). The telomere probe was added to digested genomic DNA and heated at 98°C for 6 minutes followed by hybridization for 2 hours at 55°C. Samples were cooled to 4°C, loaded onto a 0.8% agarose gel, and electrophoresed for 18 hours. Gels were dried at room temperature wrapped in plastic and exposed to PhosphorImaging Cassettes. Average telomere lengths were determined using ImageQuaNT Software (Molecular Dynamics) (Ouellette et. al., 2000).

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Figure Legends

Figure 1. Treatment of Prostate Cancer Cells with Radicicol Transiently Inhibits Telomerase Activity and Reduces Telomere Length, Culminating in Apoptosis. (A) Genomic DNA was isolated from cells exposed to chronic RAD and digested with 6 restriction enzymes. The digested DNA was hybridized with a ^{32}P-labeled telomere probe, and telomere signals separated by gel electrophoresis. Note the 1.5kb shortening over 16 days. **(B)** Cells continuously cultured in 0.3μM RAD or vehicle (DMSO) were assessed for cell growth in log phase over the course of treatment. **(C)** Following chronic RAD treatment for 55-days, cells were stained with terminal transferase and Fluoroscein 12-dUTP (TUNEL) and DAPI (not shown). All DAPI-positive RAD-treated cells were stained with TUNEL, visualized by an intense fluorescent (green) signal, while the DMSO control were not TUNEL positive. **(D)** The M12 prostate cancer cells treated with 0.3μM Radicicol or DMSO were periodically analyzed for telomerase activity (100 cell equivalents) by the TRAP assay. IC is the 36bp internal PCR control. **(E)** 10μg total protein prepared from cells exposed to chronic RAD or DMSO were subjected to immunoblotting with an anti-ubiquitin antibody to visualize all ubiquitin-tagged proteins.

Figure 2 Radicicol Induces NOS-Dependent Free Radicals. (A) RAD induces a dose dependent increase in superoxide (O^{2-}) generation in the M12 metastatic prostate cancer cells. Cells were incubated in fresh media containing DHE for 30 minutes, followed by imaging using a fluorescent microscope. Cells generating high concentrations of free radicals emit an intense red fluorescence. **(B)** Cells chronically treated with RAD or DMSO for 55 days were reseeded into 8-well chamber slides, incubated with DHE for 30 minutes, and visualized using a fluorescent microscope. Note the high concentrations of free radicals in RAD-treated cells

compared to the vehicle controls (DMSO). **(C)** Cells were incubated in 0.3μM or 1μM RAD for 24hrs with or without 500μM of the NOS inhibitor L-NAME, and then incubated in fresh media containing DHE for 30 minutes. Free radical production was quantified by flow cytometry analysis and plotted on a logarithmic scale.

Figure 3. Telomere Regrowth and Prevention of Apoptosis in Cells after Radicicol is Removed. **(A)** Growth of M12 cells continuously cultured in presence of RAD for 49 days. At day 50, cells were either maintained in RAD media (or DMSO) or removed from drug (or vehicle control). Growth was calculated as population doublings from logarithmically dividing cells. **(B)** RAD was removed at day 49, and cells were cultured for the indicated days beyond drug (or vehicle) removal. Genomic DNA was isolated at the indicated times, and subjected to the TALA protocol to assess telomere length. The average telomere length (Q:) was measured using ImageQuant software as before (Ouellette et. al., 2000). Note the increase in telomere length after RAD removal, as indicated by the white bars.

Figure 4. Inhibition of the NOS Pathway Prevents Telomere Shortening and Apoptosis in RAD-treated Cells. Cells were cultured for 44 days in RAD media. On day 45, cells were either maintained in RAD alone (or DMSO) or co-cultured in L-NAME (or DMSO) as indicated. Cells were assessed for **(A)** growth over time and **(B)** telomere length, determined using TALA.

Figure 5. Genetic Inhibition of Hsp90 Causes Telomere Shortening without Altering Telomerase Activity or Cell Growth. **(A)** Mass cultures of M12 cells with siRNA expression specific for either Hsp90α or both Hsp90 isoforms (α and β), along with vector (pSUPER)

controls, were tested for expression Hsp90 by immunoblotting. Note: no change in Hsp90 expression with the siRNA specific for the α isoform. **(B)** Hsp90α protein levels following stable integration of the Hsp90 and Hsp90α siRNA constructs. **(C)** Hsp90 expression in single cell-derived clones (1 and 2) after infection with siRNA directed at both Hsp90 isoforms. **(D)** Telomerase activity was tested in M12 mass cultures (left panel) and clones (right panel) following infection with siRNA constructs directed at Hsp90. Samples of 100,000 cells were collected and cell lysate aliquots corresponding to 500 cell equivalents were analyzed for telomerase activity by the TRAP assay. Relative telomerase activity can be visualized using signal intensity of the telomerase ladder compared to the internal control (IC). **(E)** Growth of logarithmically cultured M12 cells following Hsp90 siRNA expression was calculated. **(F)** Telomere lengths of individual clones were analyzed using 20μg of genomic DNA and the TALA protocol as in Figure 1. The average telomere length (Q) was measured as before (Ouellette et. al., 2000).

Figure 6. Inhibition of NOS-induced Free Radicals Prevents Telomere Erosion. (A) Cells expressing the vector (pSUPER) or siRNAs against total Hsp90 or Hsp90α were incubated in media containing DHE for 30 minutes. After dye removal, DHE stained cells were visualized using a fluorescent microscope (increased superoxide levels are visualized by an intense red fluorescence). **(B)** Hsp90 siRNA-expressing cells were incubated in fresh media (or media containing L-NAME for 24 hours) and then with media containing DHE dye for 30 minutes. Free radical production as a measure of dye uptake was analyzed by flow cytometry. **(C)** Telomere lengths were analyzed over time in cells expressing Hsp90 siRNAs and compared

relative to the pSUPER controls. Genomic DNA was isolated from cells after incubation with 500mM L-NAME and subjected to the TALA.

Figure 7. Model of Hsp90-Mediated Regulation of Nitric Oxide Synthase (NOS) and Free Radical-Induced Telomere Damage. NOS produces nitric oxide (˙NO) as a byproduct of the reaction in which L-Arginine and oxygen are converted to L-Citrulline. Hsp90 association with NOS promotes this conversion and suppresses the pathway by which NOS generates the superoxide (O^{2-}) free radicals. Inhibition of Hsp90 function pharmacologically (GA or RAD) or genetically (siRNA) disrupts NOS conformation to inhibit ˙NO production and promote O^{2-} generation, which results in the accumulation of telomere damage (shortening), rendering the cell susceptible to apoptosis. L-NAME, by blocking both NOS pathways, protects against telomere damage and apoptosis (not depicted in the model).

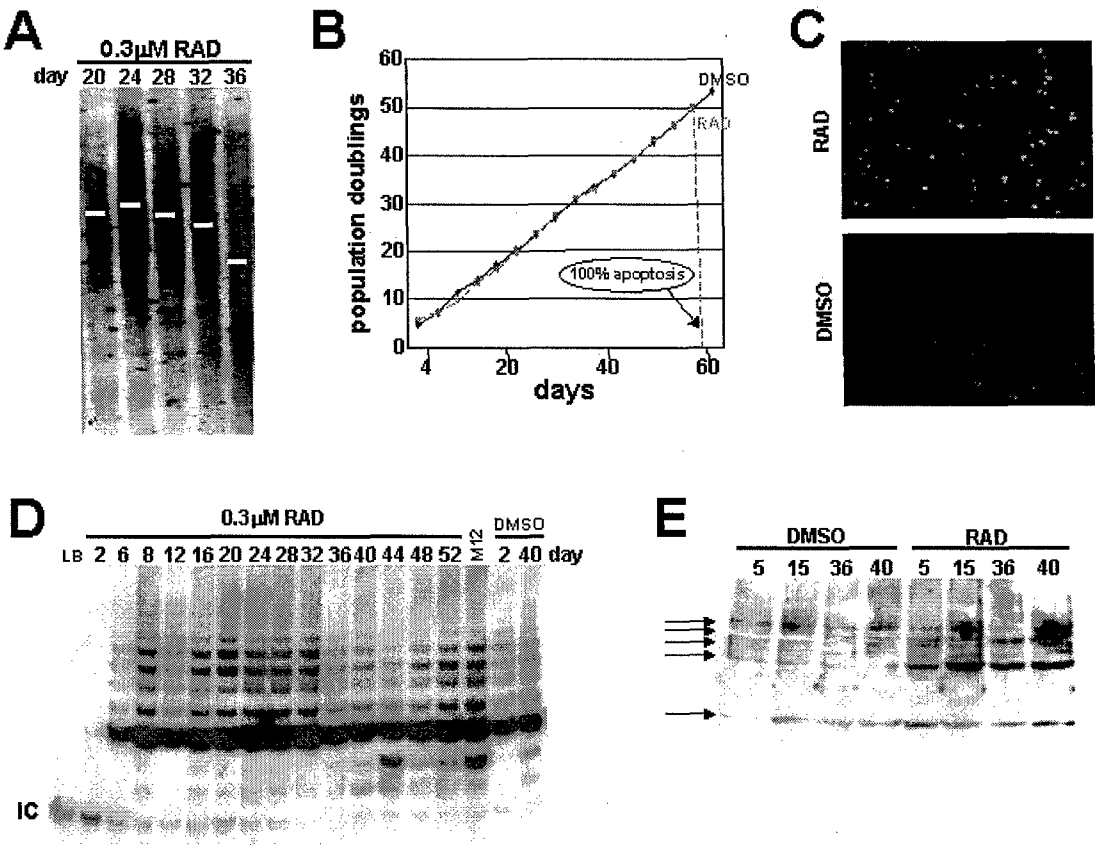
A
0.3μM RAD
day 20 24 28 32 36
B
population doublings
60
50
40
30
20
10
0
DMSO
RAD
100% apoptosis
4
20
40
60
days
C
RAD
DMSO
D
0.3μM RAD
LB 2 6 8 12 16 20 24 28 32 36 40 44 48 52 M12
DMSO
2 40 day
IC
E
DMSO
RAD
5 15 36 40 5 15 36 40

Figure 1

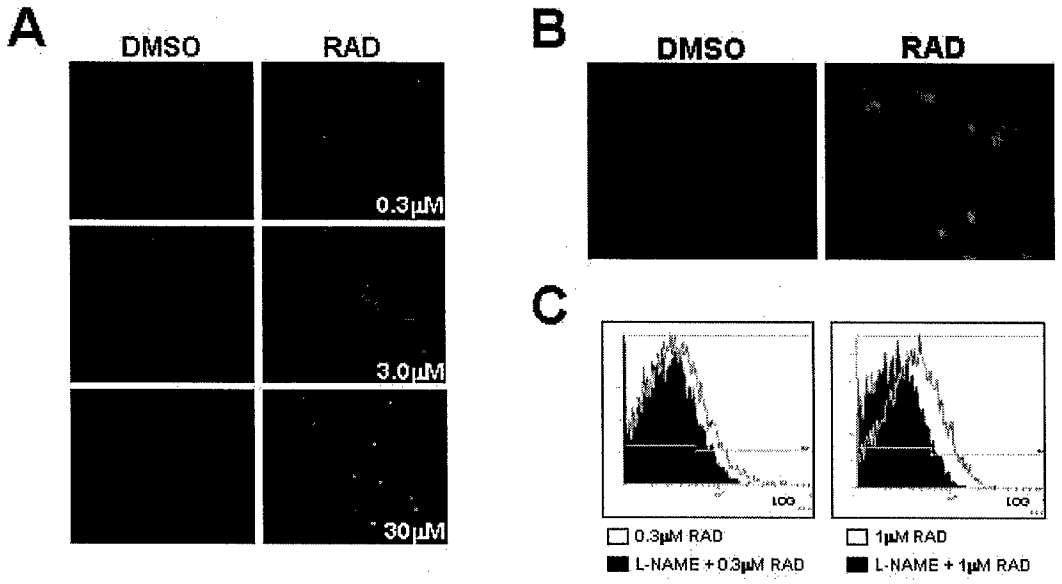
A
DMSO
RAD
0.3μM
3.0μM
30μM
B
DMSO
RAD
C
LOG
LOG
0.3μM RAD
L-NAME + 0.3μM RAD
1μM RAD
L-NAME + 1μM RAD

Figure 2

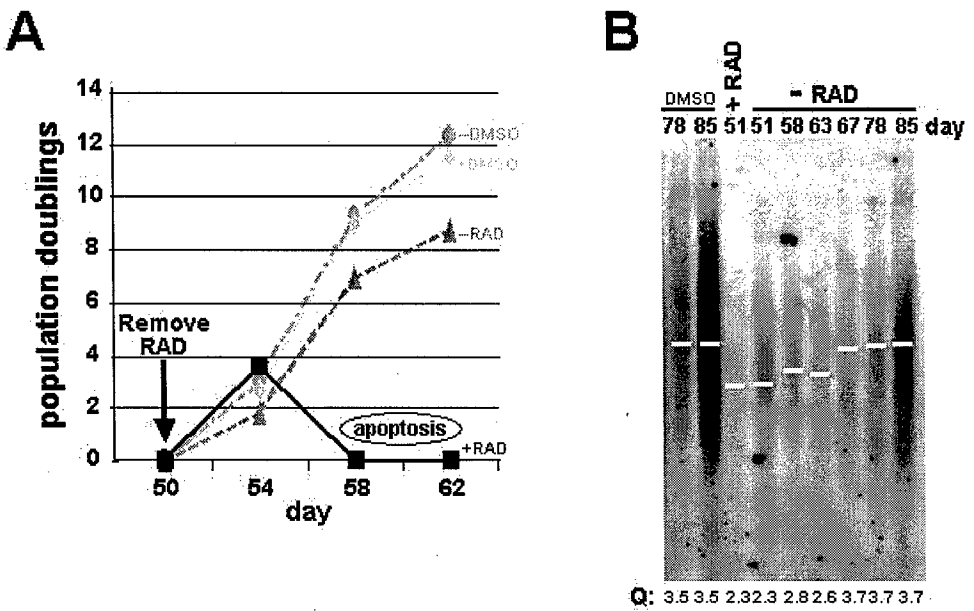
A
population doublings
14
12
10
8
6
4
2
0
Remove RAD
apoptosis
+RAD
-RAD
50
54
58
62
day
B
DMSO
+ RAD
- RAD
78 85 51 51 58 63 67 78 85 day
Q: 3.5 3.5 2.3 2.3 2.8 2.6 3.7 3.7 3.7

Figure 3

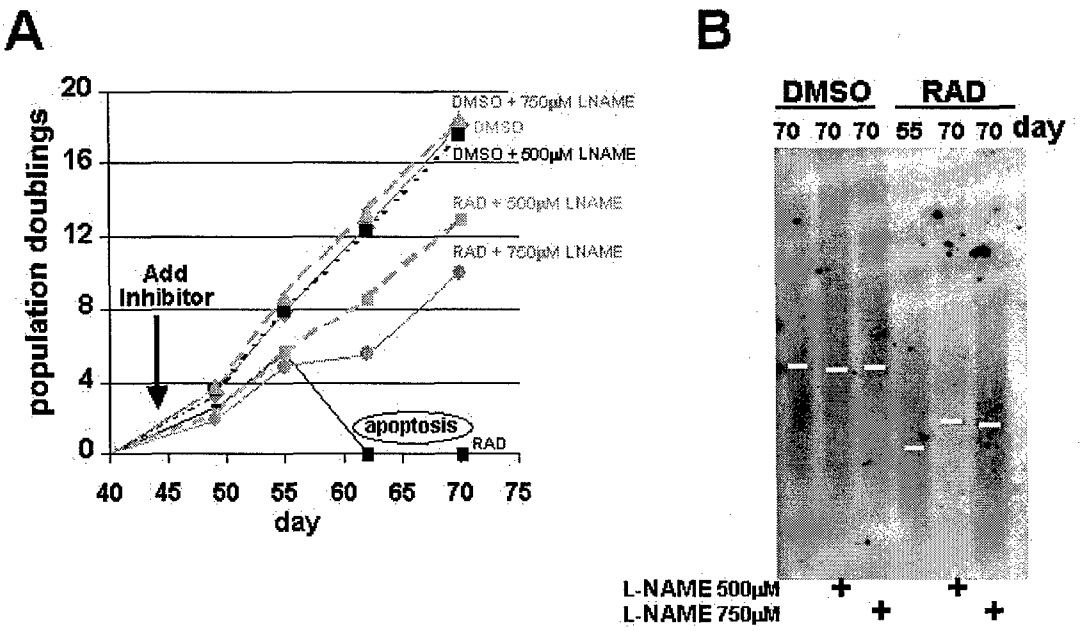
A
B
population doublings
20
16
12
8
4
0
40 45 50 55 60 65 70 75
day
Add Inhibitor
DMSO + 750μM LNAME
DMSO
DMSO + 500μM LNAME
RAD + 500μM LNAME
RAD + 750μM LNAME
apoptosis
RAD
DMSO
RAD
70 70 70 55 70 70 day
L-NAME 500μM
L-NAME 750μM

Figure 4

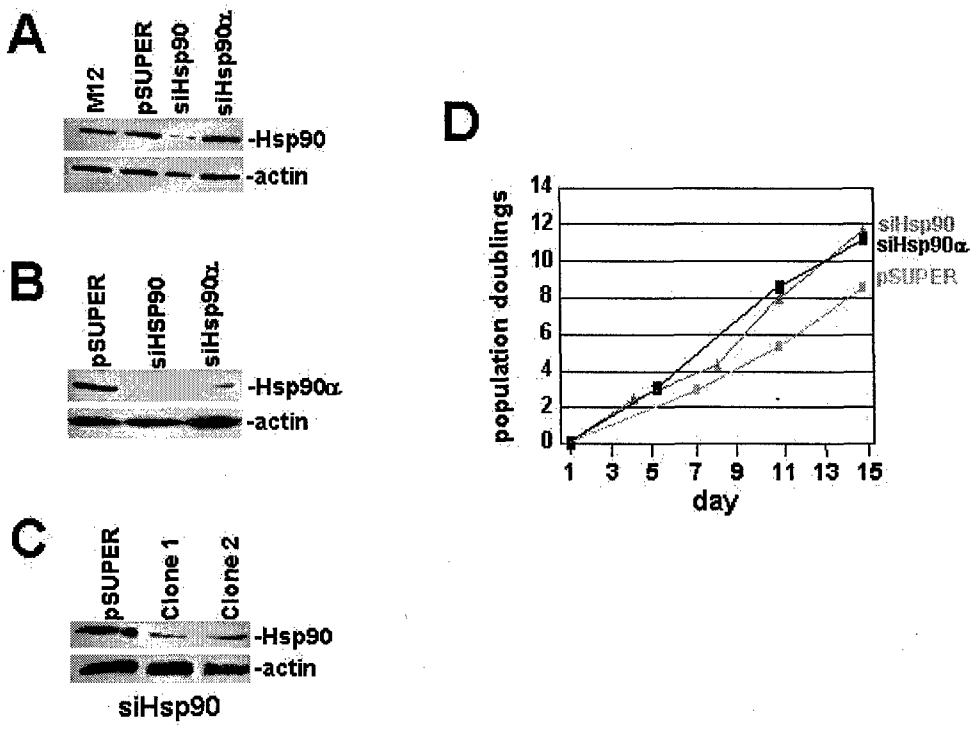
A
M12
pSUPER
siHsp90
siHsp90α
-Hsp90
-actin
B
pSUPER
siHSP90
siHsp90α
-Hsp90α
-actin
C
pSUPER
Clone 1
Clone 2
-Hsp90
-actin
siHsp90
D
population doublings
14
12
10
8
6
4
2
0
1
3
5
7
9
11
13
15
day
siHsp90
siHsp90α
pSUPER

Figure 5

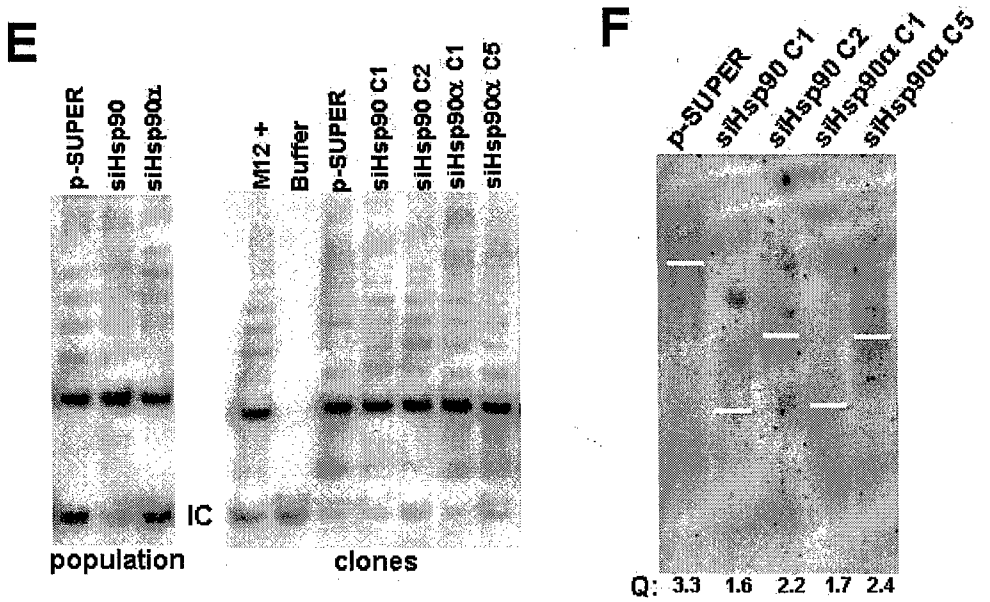
E
p-SUPER
siHsp90
siHsp90α
IC
population
M12 +
Buffer
p-SUPER
siHsp90 C1
siHsp90 C2
siHsp90α C1
siHsp90α C5
clones
F
p-SUPER
siHsp90 C1
siHsp90 C2
siHsp90α C1
siHsp90α C5
Q: 3.3 1.6 2.2 1.7 2.4

Figure 5

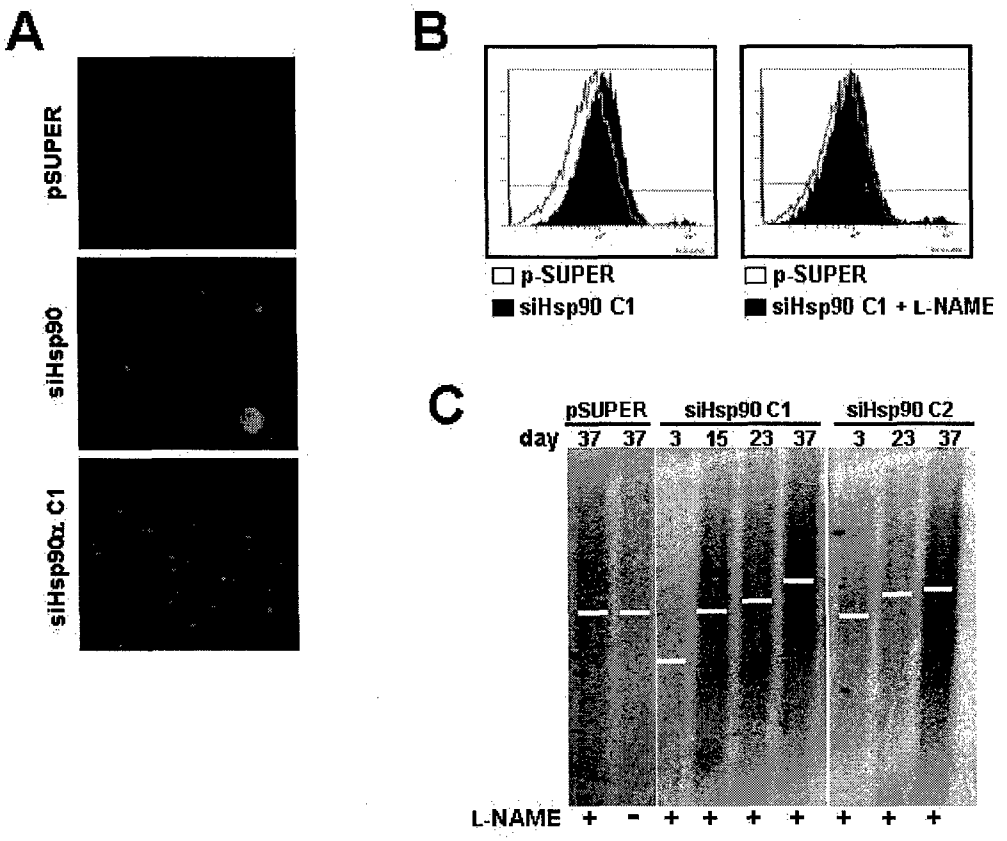
A
pSUPER
siHsp90
siHsp90α C1
B
p-SUPER
siHsp90 C1
p-SUPER
siHsp90 C1 + L-NAME
C
pSUPER
siHsp90 C1
siHsp90 C2
day
37 37 3 15 23 37 3 23 37
L-NAME
+ - + + + + + + +

Figure 6

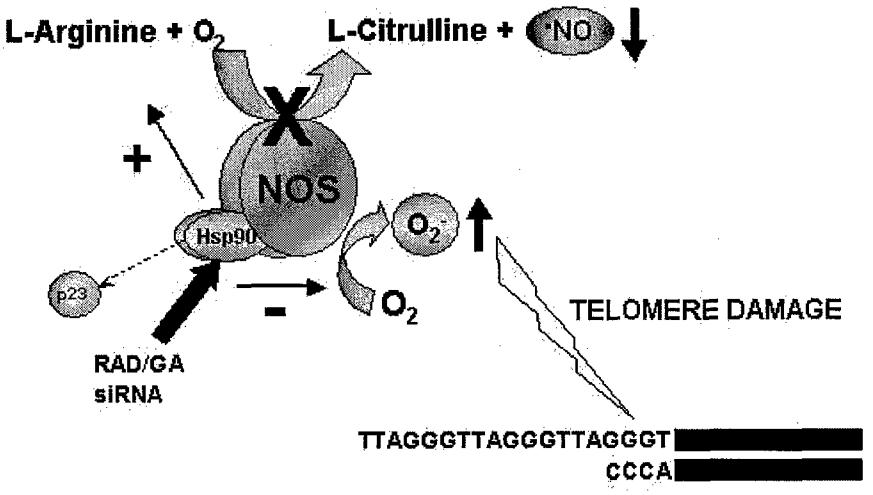
L-Arginine + O_2
L-Citrulline + NO
X
NOS
+
Hsp90
p23
O_2^-
O_2
-
RAD/GA
siRNA
TELOMERE DAMAGE
TTAGGGTTAGGGTTAGGGT
CCCA

Figure 7